



Degradability of *n*-alkanes during *ex situ* natural bioremediation of soil contaminated by heavy residual fuel oil (mazut)

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Abstract: It is well known that during biodegradation of oil under natural geological conditions, or oil pollutants in the environment, degradation of hydrocarbons occurs according to a well-defined sequence. For example, the major changes during the degradation process of *n*-alkanes occur in the second, slight and third, moderate level (on the biodegradation scale from 1 to 10). According to previous research, in the fourth, heavy level, when intensive changes of phenanthrene and its methyl isomers begin, *n*-alkanes have already been completely removed. In this paper, the *ex situ* natural bioremediation (non-stimulated bioremediation, without addition of biomass, nutrient substances and biosurfactant) of soil contaminated with heavy residual fuel oil (mazut) was conducted during a period of 6 months. Low abundance of *n*-alkanes in the fraction of total saturated hydrocarbons in the initial sample (identification was possible only after concentration by the urea adduction technique) showed that the investigated oil pollutant was at the boundary between the third and the fourth biodegradation level. During the experiment, an intense degradation of phenanthrene and its methyl-, dimethyl- and trimethyl-isomers was not accompanied by the removal of the remaining *n*-alkanes. The abundance of *n*-alkanes remained at the initial low level, even at end of the experiment when the pollutant reached one of the highest biodegradation levels. These results showed that the non-stimulated biodegradation of some hydrocarbons, despite their high biodegradability, had not proceeded completely to the end, even at final degradation stages. Under the condition of reduced availability of some hydrocarbons, microorganisms tend to opt for the less biodegradable but more accessible hydrocarbons.

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INTRODUCTION

Biodegradation and the removal of oil-type pollutants (crude oil and refinery products of petroleum refining) from the environment are difficult to be classified in one category. Oil is a very complex mixture of hydrocarbons, but also nitrogen, sulfur and oxygen compounds (NSO). Each class of compounds, and often individual compounds as well, require special study aimed at defining the type of microorganisms and optimal conditions for microbial degradation.^{1,2}

Knowledge about the rate of microbial degradation of individual organic compounds in oil is mostly based on organic geochemical research. As early as 1984, Volkman *et al.* classified oils into 9 groups according to their degree of biodegradation.³ Biodegradation begins with the degradation of lower *n*-alkanes ($< n\text{-C}_{15}$), while maximum degraded oils, with completely degraded steranes and dominated by diasteranes and demethylated hopanes, belong to the ninth group. In their research, these authors interpreted biodegradation of oil mainly through the biodegradation of *n*-alkanes, isoprenoid alkanes and polycyclic alkanes.

In 2003, Head *et al.* completed the classification of oils according to the biodegradation level, comparing the degradability of a larger number of classes of organic compounds.⁴ First, they included mono- and tri-aromatic steroids as well as phenanthrene with its methyl isomers. According to these authors as well, biodegradation of *n*-alkanes starts on the first level. Only in the last phases, do the degradation of mono- and tri-aromatic steroids, gammacerane, oleanane, C_{21} and C_{22} steranes, tricyclic terpanes, diasteranes, diahopanes and norhopanes begin. Among these compounds, only C_{21} and C_{22} steranes and tricyclic terpanes can be removed completely.

For the degradation of some compounds, microorganisms and conditions for degradation are unknown, and these compounds are considered non-biodegradable. This is especially true for compounds in the fraction of NSO-compounds.⁵

In this study, *ex situ* natural microbial degradation of soil contaminated with heavy residual fuel oil (mazut) was conducted during a period of 6 months. The fate of the *n*-alkanes in the pollutant was monitored. Namely, the main transformations during the degradation process of *n*-alkanes, which are the dominant hydrocarbons in most oils and oil-type pollutants, occur during the second, slight and the third, moderate level (on the biodegradation scale from 1 to 10).⁴ According to previous research, in the fourth, heavy level, when intensive changes of phenanthrene and its methyl isomers begin, the *n*-alkanes had already been completely removed. However, in the samples investigated in the present study, at the very beginning of the biodegradation experiment, *n*-alkanes within the fraction of saturated hydrocarbons were present in amount close to the detec-

tion limit. The question arose as to what their fate would be in case of their minimum availability to the microorganisms.

EXPERIMENTAL

The *ex situ* natural biodegradation (non-stimulated bioremediation, without addition of biomass, nutrient substances and biosurfactant) of a soil contaminated with heavy residual fuel oil (mazut) was conducted. The crude oil-polluted soil was excavated contaminated soil from an energy power plant that, due to a breakdown, had been polluted for a year with heavy fuel oil and sediment from a heavy oil reservoir.

The crude oil-polluted soil (approximately 150 t; 210 m³) was uniformly distributed over 300 m³ of not rinsed sand from the Sava River (settlement Ostruznica, Serbia). Sawdust from poplar, beech and oak (approx. 60 m³) was added in order to not only increase the retention water capacity, but also as an alternative, additional carbon (C) substrate. The entire material (volume of approx. 600 m³), defined as the substrate for biodegradation, was homogenized, and then formed into a biopile shape with dimensions of 75 m×20 m×0.4 m (length, width, height), with bulldozers. Immediately after mixing, approximately 10 m³ of the biopile mixture was set aside on the same waterproof asphalt surface to be used as a substrate for monitoring the natural biodegradation. The experiment was conducted in autumn and winter with average daily temperatures ranging from 25 to –10 °C. However, due to the intensive microbiological activity, the temperature of the soil was stable, above 25 °C. After mixing, the biopile was covered with polyethylene foil to prevent loss of temperature and the direct influence of the weather conditions on the bioremediation substrate.

From the polluted soil, a consortium of hydrocarbon degrading microorganisms was isolated and individual components were identified. Analytical Profile Index (API-Biomerieux) tests were conducted with isolated cultures of the microorganisms and the following were identified: *Pseudomonas aeruginosa*, *Rhodococcus* sp., *Pseudomonas* sp., *Pseudomonas fluorescens*, *Sphingomonas paucimobilis*, *Pseudomonas luteola*, *Achromobacter denitrificans*, *Stenotrophomonas maltophilia* and *Aeromonas hydrophila*. The number of microorganisms was determined by plating appropriate serial dilutions on agar plates incubated at 28 °C. The media used were nutrient agar for total chemoorganoheterotrophs (TC) and a mineral base medium⁶ containing 2 g of standard D2 diesel fuel in 1 L of medium⁷ for hydrocarbon degraders (HD). The results are presented in Table I. More detailed characteristics of the investigated mazut-polluted soil were presented in a previous paper.⁸

TABLE I. Content of microorganisms (CFU g⁻¹) in the investigated oil-polluted sample; TC – total chemoorganoheterotrophs; HD – hydrocarbon degraders; CFU – colony-forming unit

Specimen	Polluted soil	Substrate for biodegradation
TC	1.2×10 ⁶	9.7×10 ⁵
HD	2.7×10 ⁵	5.6×10 ⁴

During six-month time interval, samples were taken five times (07/09/2009 – P1; 06/10/2009 – P2; 09/11/2009 – P3, 12/01/2010 – P4 and 18/03/2010 – P5).

The organic substance from the 5 soil samples was extracted with chloroform (HPLC grade, J. T., USA) using a Soxhlet apparatus.

From these extracts, the hydrocarbons (saturated and aromatic) were isolated by column chromatography: the extracts were saponified with a 5 % solution of KOH in methanol and after standing overnight, neutralized with 10 % hydrochloric acid. The products were dis-

solved in a mixture of dichloromethane (containing 1 % methanol) and hexane (1:40), and separated by column chromatography on alumina and silica gel. The hydrocarbon fractions were eluted with hexane (saturated hydrocarbons) followed by dichloromethane (aromatic hydrocarbons). A detailed description of the analytical procedure was discussed in previous papers.^{9,10}

Hydrocarbons were analyzed by the gas chromatography–mass spectrometry (GC–MS) techniques. An Agilent 7890N gas chromatograph fitted with a HP5-MS capillary column (30 mm×0.25 mm, 0.25 µm film; temperature program: 80 °C for 0 min; then 2 °C min⁻¹ to 300 °C and held for 20 min) with helium as the carrier gas (flow rate 1 cm³ min⁻¹) was used. Detailed analyses of the target compounds were conducted in the single ion monitoring mode (SIM), comprising the following ion chromatograms: 178 (phenanthrene), 192 (methyl-phenanthrenes), 206 (dimethyl-phenanthrenes) and 220 (trimethyl-phenanthrenes). A detailed description of the instrumental techniques was discussed in a previous paper.¹¹

The saturated hydrocarbon mixture was separated into *n*-alkane and branched and cyclic alkane fractions by urea adduction.¹² The *n*-alkanes in the urea adducts were analyzed by gas chromatography (GC). The gas chromatographic analyses were conducted on a GC8000 instrument (Fisons Instruments, Italy) equipped with a 30 m×0.25 mm i.d.×0.25 µm film ZB1 fused silica capillary column (Phenomenex, Germany). The chromatographic conditions were as follows: 1 µL split/splitless injection at 80 °C oven temperature (injector temperature 270 °C, splitless time 60 s), 3 min hold, then programmed at 5 °C min⁻¹ to 300 °C. The carrier gas was hydrogen at a velocity of 40 cm s⁻¹.

RESULTS AND DISCUSSION

Total ion chromatograms (TICs) of saturated hydrocarbon fractions isolated from the oil pollutant at the beginning of the biodegradation (P1) and after 6 months (P5), at the end of the experiment, are shown in Fig. 1. In similar samples, P1 and P5, the peaks originating from *n*-alkanes were of very low intensity. Based on these chromatograms, a precise conclusion about the extent of microbial degradation of the investigated oil pollutant could not be made.

Mass fragmentograms of phenanthrene, methyl-, dimethyl- and trimethyl-phenanthrenes obtained by GC–MS analysis of aromatic fractions isolated from extracts of samples P1–P5 are shown in Fig. 2. In the initial sample (P1), the distributions of these aromatic hydrocarbons were typical for oil. Considering the fact that most of *n*-alkanes in the initial sample have already been degraded (Fig. 1), and that phenanthrene and its methyl-isomers were still preserved, it could be concluded that the investigated sample of oil pollutant was at the third, moderate level of the Head scale of oil biodegradation at the beginning of the experiment.⁴

In the samples from P1 (the beginning of *ex situ* natural bioremediation, 7th September 2009) to P5 (the end of the experiment, 18th March 2010), a gradual degradation of phenanthrene and its methyl-, dimethyl- and trimethyl-isomers was registered (Fig. 2). On the scale from 1 to 10 of the Head classification of oils according to biodegradation level,⁴ sample P5 can be classified as > 6, the severe level.

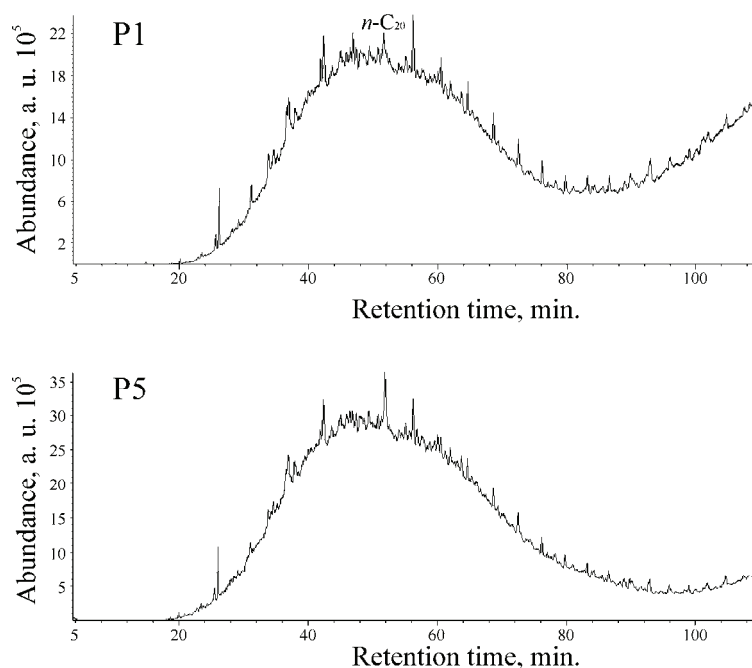


Fig. 1. Total ion chromatograms (TICs) of the saturated hydrocarbon fractions isolated from the oil pollutant. P1 (beginning of the experiment) and P5 (after the six-month bioremediation).

Considering the fact that in the initial sample, the amount of *n*-alkanes within the saturated hydrocarbons fraction was very low (Fig. 1), these compounds were concentrated by urea adduction technique to facilitate their analysis. Due to their ability to form channel inclusion compounds with urea molecules, *n*-alkanes can be successfully concentrated, *i.e.*, separated from branched, cyclic and polycyclic alkanes in a mixture of saturated hydrocarbons.¹²

Parallel with the mass fragmentograms of phenanthrene and its methyl-isomers, gas chromatograms of the urea adduct from samples P1–P5 are given in Fig. 2. Although the amount of *n*-alkanes within the total mixture of saturated hydrocarbons, both at the beginning and at the end of the experiment, was very low (Fig. 1), they were successfully concentrated by the urea adduct technique. Accordingly, in the gas chromatograms of all the investigated samples, a homologous series of alkanes, ranging from C₁₆ to C₃₁ and maximizing at *n*-C₂₀, was identified. It is noticeable that the abundance and the distribution of the *n*-alkanes during the biodegradation of the oil pollutant did not change. Considering the fact that phenanthrene and its methyl-isomers, although less biodegradable compounds, were efficiently degraded from samples P1 to P5, this observation was surprising. According to the generally accepted principles of oil biodegradation,^{3,4,13} *n*-alkanes should have already been completely removed at the

biodegradation level 4 and 5.⁴ Due to this, it could be stated that their appearance in sample P5, which is obviously at a biodegradation level > 6, was unexpected.

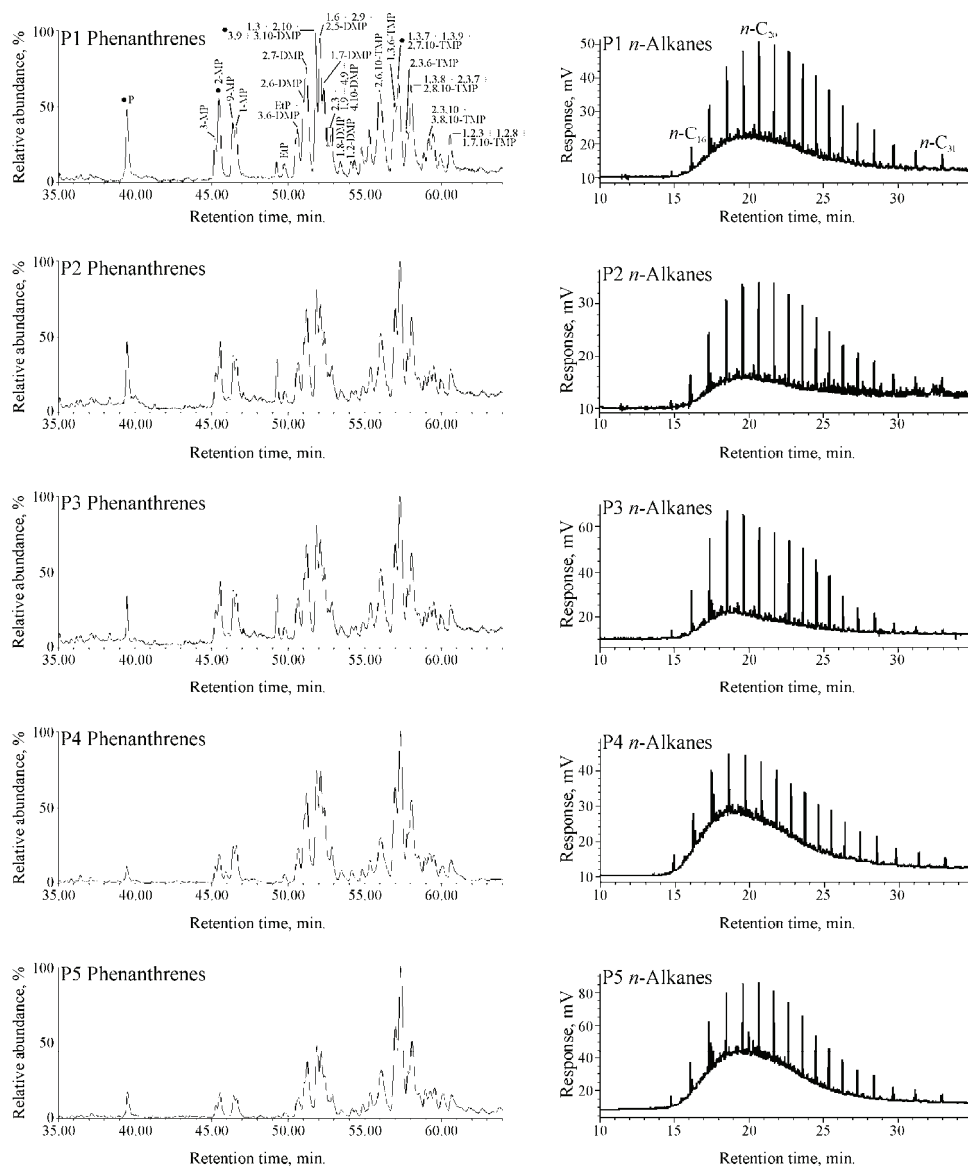


Fig. 2. Mass fragmentograms of phenanthrene (P), methyl-phenanthrenes (MP), dimethyl-phenanthrenes (DMP) and trimethyl-phenanthrenes (TMP), obtained by gas chromatography–mass spectrometry (GC-MS) analysis (using the Single Ion Monitoring, SIM method) of the aromatic fractions and the gas-chromatograms (GC) of the urea adducts of the saturated fractions (*n*-alkanes), isolated from the oil-type pollutants taken from the soil during the six-month biodegradation, P1–P5.

n-Alkanes are significantly more biodegradable compounds than aromatic hydrocarbons, including phenanthrene and its methyl-isomers. However, the results obtained in this study undoubtedly show that under conditions of reduced availability of a certain class of compounds, microorganisms opt for those that are, although less biodegradable, more accessible, *i.e.* those that are found in higher amounts in a substrate. In this case, the reason for lower biodegradability might be the smaller amounts of *n*-alkanes in the pollutant. On the other hand, this observation could also be caused by their protection by incorporation, *i.e.*, the formation of inclusions with non-biodegradable components, such as humic substances or fulvic acids, present in recent sediments as native organic compounds.

CONCLUSIONS

The *ex situ* natural biodegradation (non-stimulated bioremediation, without the addition of biomass, nutrient substances and biosurfactant) of soil contaminated with heavy residual fuel oil (mazut) was conducted during a period of 6 months. The low abundance of *n*-alkanes in the total saturated hydrocarbons fraction in the initial sample (identification was only possible after concentration by the urea adduction technique) showed that the investigated oil pollutant was at the boundary between the third and the fourth biodegradation level on the Head biodegradation scale from 1 to 10.⁴ During this experiment, intensive degradation of phenanthrene and its methyl-, dimethyl- and trimethyl-isomers was not accompanied by the complete removal of remaining *n*-alkanes. Contrary to expectations, the abundance of *n*-alkanes remained at the initial low level even at the end of the experiment when the pollutant attained a degradation level > 6.

According to these results, it can be concluded that during biodegradation of oil pollutant, under the condition of reduced availability of a certain class of compounds (caused by their low amount), microorganisms opt for those which are more accessible, *i.e.*, those which are found in a substrate in higher amounts, even if these compounds are less biodegradable.

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ИЗВОД

ДЕГРАДАБИЛНОСТ НОРМАЛНИХ АЛКАНА ЗА ВРЕМЕ *EX SITU* ПРИРОДНЕ
БИОРЕМЕДИЈАЦИЈЕ ЗЕМЉИШТА ЗАГАЂЕНОГ МАЗУТОМ

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Добро је познато да се у току биодеградације нафте у природним геолошким усло-
вима, или нафтног загађивача у животној средини, деградација угљоводоника одиграва
према дефинисаном распореду. На пример, главне промене у процесу разградње
нормалних алкана дешавају се у току другог, „благог“, и трећег, „умереног“, ступња (на
скали биодеградације од 1 до 10). Према досадашњим истраживањима, у четвртном
ступњу, када почињу интензивне промене фенантрена и његових метил изомера, нор-
мални алкани су већ у потпуности уклоњени. У овом раду у току периода од 6 месеци
извођена је *ex situ* природна биоремедијација (нестимулисана биоремедијација без до-
датка биомасе, хранљивих састојака и биосурфактаната) земљишта загађеног мазутом.
Ниска обилност нормалних алкана у фракцији укупних засићених алкана у почетном
узорку (идентификација је била могућна тек након концентровања помоћу карбамида)
показала је да је испитивани нафтни загађивач на граници између трећег и четвртог
ступња биодеградације. Током експеримента, интензивну разградњу фенантрена и њего-
вих метил, диметил и триметил изомера није пратило уклањање остатка нормалних
алкана. Њихова обилност је остала на почетном, ниском нивоу и на крају експеримента
када је загађивач достигао један од највиших степена биодеградације. Добијени резул-
тати су показали да се разградња појединих угљоводоника упркос њиховој високој био-
деградабилности не одиграва до потпуног краја ни у завршним фазама деградације. У
условима њихове смањене доступности, микроорганизми се опредељују за теже дегра-
дабилне, али доступније угљоводонике.

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